

## Can we produce tomatoes with a pinch of salt? (or how to reduce the negative impact of water and soil salinity on cultivated tomato)

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### SUMMARY

Excessive soil salinity causes abiotic stress and consequently diminishes crop yields. In this study of cultivated tomato, we analyzed one of the mechanisms of salt tolerance operating in other plant models such as *Arabidopsis* and rice. This mechanism is based on the activity of Na<sup>+</sup> transporters belonging to subfamily HKT1, which determine genetic traits associated with salt tolerance in these plant models. These transporters drive Na<sup>+</sup> through the plant to tissues and subcellular compartments where it is not toxic and/or help its expulsion from the plant. In our lab, two tomato genes encode HKT1-like transporters have been identified. To test the importance of these transporters in the mechanism of salt tolerance in tomato, we have worked with two tomato near-isogenic lines (NIL) differing in terms of the HKT gene allele they contain (from *Solanum lycopersicum* and *S. cheesmaniae*), and different transgenic lines derived from these NILs in which each one of the HKT1;1/HKT1;2 allelic variants has been previously silenced by stable gene transformation. In this study, we provide the preliminary phenotype characterization for each genotype in order to determine which of these genes/alleles plays the most significant role in tomato salt tolerance measured as tissue growth in plants cultured in different media (Petri plates, pots and hydroponics). Results obtained may be the basis for future research in order to improve the tolerance of plant crops to salinity in water and soils.

### INTRODUCTION (AND OBJECTIVES)

On a world scale, no toxic substance restricts plant growth more than salt. More than 800 million hectares of land throughout the world are salt affected [1]. In Spain soil salinity is a very serious problem for a great variety of crops, including tomato. High salt concentrations in the root growth media impose both an ionic and an osmotic stress to most plants [2]. The major ionic stress associated with high salinity is due to sodium (Na<sup>+</sup>) toxicity. Under salinity conditions, Na<sup>+</sup> is taken up by roots, transported to shoots in the transpiration stream and accumulated in plant cells over time [2,3]. Because of its toxicity, Na<sup>+</sup> accumulation in the cytosol of plant cells results in progressive damage affecting negatively many physiological processes within the plant [2]. In addition, a high external Na<sup>+</sup> concentration also prevents the uptake K<sup>+</sup>, leading to insufficient cellular K<sup>+</sup> amount for enzymatic reactions and osmotic adjustment [2,3]. Therefore the regulation of intracellular concentration of Na<sup>+</sup> and K<sup>+</sup> (homeostasis) in plant cells and tissues is a key mechanism in saline stress tolerance [3]. All these involves a network of processes regulating uptake, extrusion through the plasma

membrane, compartmentation of salts into cell vacuoles and recirculation of ions through the plant organs, thus allowing the osmotic adjustment and maintenance of high  $K^+/Na^+$  ratios in the cytosol of plants grown under salt stress. In order to control  $Na^+$  (and  $K^+$ ) homeostasis, plants have different  $Na^+$  transporters to protect the plant against damage due to  $Na^+$  accumulation: antiporters in the root that extrude  $Na^+$  back to the soil in a mechanism coupled to  $H^+$  transport (involving the SOS pathway) [4-7]; transporters that retrieve  $Na^+$  from the transpiration stream avoiding the over-accumulation of  $Na^+$  in the photosynthetic tissues (involving HKT transporters) [8-10]; and antiporters that sequester  $Na^+$  in the vacuoles (involving NHX1 antiporters), along the electrochemical gradient generated by the  $H^+$ -ATPase and the  $H^+$ -PPase [2,11].

HKT1-like transporters are one of the most studied  $Na^+$  permeable transporters which play an important role in  $Na^+$  and  $K^+$  homeostasis [9]. These  $Na^+$  transporters, located at plasma membrane of parenchyma cells surrounding the xylem vessels, are responsible for unloading  $Na^+$  from the xylem, thus preventing  $Na^+$  accumulation in aerial parts and indirectly improving  $K^+$  homeostasis in many plant species [8-10]. Recent studies have shown their crucial importance in salinity tolerance in both mono- and dicotyledonous species [10,12,13]. This makes HKT transporters a preferential target for the engineering of plant stress tolerance. However, salt tolerance in plants is a quantitative trait that could be determined by one or multiple genes. The identification of quantitative trait loci (QTLs) controlling this characteristic is of great importance in order to breed salt-tolerant crops [14, 15]. Using two tomato near-isogenic lines (NIL) differing in terms of the HKT gene allele they contain (from *Solanum lycopersicum* and *S. cheesmaniae*) showed that the connection between the allelic variants of tomato HKT1;1 and HKT1;2 and salt tolerance was unclear and mostly depended on salt tolerance criteria used [16]. Therefore, to test the above hypothesis and whether these transporters are important in the mechanism of salt tolerance in tomato we previously generated different transgenic lines derived from these NILs in which each one of the HKT1-like allelic variants has been previously silenced by stable gene transformation (Belver et al., unpublished results).

In this study, we provide the preliminary phenotype characterization for each genotype in order to determine which of these genes/alleles plays the most significant role in tomato salt tolerance measured as tissue growth in plants cultured in different media (Petri plates, pots and hydroponics).

## **MATERIALS AND METHODS**

### **Plant material**

We used two types of tomato seeds called NIL 14 and NIL 17 (Near-isogenic lines) only differing in terms of HKT allele. NIL 14 contains the HKT1-like alleles from the wild salt tolerant *S. cheesmaniae* and NIL 17 contains the *S. lycopersicum* alleles (supplied by Dr. MJose Asins, IVIA Valencia). The *S. cheesmaniae* allele makes it possible to store more sodium and less potassium in the aerial part of the plant when cultivated under saline conditions [15,16]. Given that RNAi silencing constructs are dominant traits, different silenced T1 lines of each HKT1-like gene were used (generated in collaboration with Dr Vicente Moreno's lab, IBMCP-CSIC-Univ. Politecnica de Valencia). As a control line, we used the non-silenced HKT Ti14 and Ti17 lines, which were also subjected to the whole gene transformation process.

### **Tomato plant growth conditions**

Phenotypic evaluation of plants was performed using seedlings grown in solid medium in Petri plates, as well as plants grown in hydroponics and in pots. Seeds were sterilized by immersing

them in an ethanol solution for two minutes in order to remove the gelatinous layer on the seed. Ethanol was eliminated and the seeds were washed using distilled water and were then immersed in a bleach solution for 20 minutes. The seeds were washed 4 times in sterile water and were left to soak all night at 25 °C. Finally, the seeds were stored at 4 °C for 24 hours. This last step was carried out in order to stimulate germination uniformly.

After sterilization, seeds were cultivated using 3 different techniques: Petri plates (non-transpiring conditions), pots and hydroponics (transpiring conditions).

**Petri Plate culture:** tomato seeds used were surface sterilized and germinated in Petri plates (10x10 cm) containing MS medium [17]. Cultivation was performed in an environmentally controlled chamber at 24°C/18 °C day/night and a 16-h light/8-h dark cycle with irradiation of  $140 \mu\text{mol m}^{-2} \text{s}^{-1}$ . The seedlings were kept in these conditions for 5 days, after which it was transferred in sterile conditions in a laminar flow chamber to new plates containing MS medium supplemented with 150 mM NaCl for five additional days. We took pictures and we analyzed the fresh weight of aerial part and roots separately and determined dry weight.

**Pot culture:** With this technique we pretended growing plants in a similar way to normal conditions, using cocopeat, an inert substrate, as support for the plant. Apart from irrigation, in the greenhouse, we also controlled light conditions, temperature and humidity, so that experimental conditions could be reproducible for all experiments. These experimental conditions are normally very similar to the ones that are used normally in tomato culture before marketing. Sterilized seeds were grown individually and kept in a culture chamber at 25°C in darkness and irrigated with water until the emergence of the cotyledons of the plant (5-7 days). Later, each plant where transferred to a pot with a cocopeat. They were cultivated in a greenhouse with a natural light irradiation supplemented with artificial light of  $122 \mu\text{moles m}^{-2} \text{s}^{-1}$ , with a relative photoperiod, temperature and humidity of 16/8 hours, 24 °C/18 °C and 40/55%, day/night, respectively. Watering was applied 2-3 times a week with a ¼ Hoagland solution [18]. When plants were in the vegetative stage of 6-leaves, we applied the saline treatment, consisting in irrigation containing 100mM of NaCl, during 15 days. We cultivated 6 pots per line with a plant per pot, three of them receiving the saline treatment and the others three only nutrient solution (control treatment). Growth analysis was monitored by photographic record, and the determination of the fresh and dry weight of the stem and leaves.

**Hydroponics culture:** This is a method where a liquid media with solution of mineral salts is used instead of soil. With this system is easier to control the different treatments we need to apply in our experiments and the root of the plant roots keeps clean and easy to sample. Sterilized tomato seed, were germinated in plastic boxes containing quartz sand sterile (inert support) for 5-7 days in darkness and at 24°C. Germinated seeds were cultivated in a growth chamber, with controlled temperature and humidity conditions. Seedlings were watered for one week with a 1/10 dilution of Hoaglands nutrients solution [18] and for another week with a ¼ dilution of the same nutrient solution. Four-leaves seedlings were transferred to 2,5-L pots (three plants for pots) and grown in a greenhouse under same conditions indicated for pots, in hydroponic system for 15 days in an aerated ¼ dilution of Hoagland solution, that was renovated every three days to avoid contamination. Ten days after hydroponic culture initiation, we applied the saline treatment, by adding 100 mM NaCl to the new ¼ dilution nutrient solution, the plants growing on it for 6 additional days. We used 2 pots with 3 plants per line), two of them receiving the saline treatment and the other ones only nutrient solution (control treatment). The growth analysis were carried out as for pot culture.

**Determination of tissue fresh and dry weight**

We collected tissue samples from leaves, stems and roots of each plant after treatment. each sample was washed four consecutive times in deionized water to eliminate salt adhered to the surface of the tissues and we dried out with filter paper. Tissue samples were weighed in a balance to determinate the fresh weight. Each sample was oven dried at 70 °C for 48 hours between filter papers and weighed in a balance to obtain the dry weight.

**Determination of Na<sup>+</sup> and K<sup>+</sup> content**

We intended to determine the content of Na<sup>+</sup> and K<sup>+</sup> in the dry material but, unfortunately, we could not do it because of ICP-OE Service from EEZ was provisionally out of service.

**RESULTS AND DISCUSSION**

The results obtained in this preliminary assessment of the phenotype for HKT-silenced tomato lines were very interesting and will be confirmed in future experiments. These findings will be the basis for future research in order to improve the tolerance of plant crops to salinity in water and soils.

Data obtained in this project are not provided here as the research carried out is subject to the confidentiality rules imposed by the ongoing R&D National Project as well as a doctoral thesis. In addition, the materials used in this research are the result of collaborative work with two external laboratories, which are also subject to confidentiality rules and copyright. However, such research has proved useful to show students the overall objective of this PIISA Project: “to show students interested in scientific research (or to make them interested in) what RESEARCH is and how it is carried out”. Nevertheless, details of these results will be presented as an oral presentation and in poster form.

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## MY OWN IDEAS

**Sofía Clemente Olías**, CDP Sagrado Corazón de Jesús, Granada

This Project has shown me how to work as a scientist such as thinking like one, organizing myself like one and working in a laboratory with a lot of scientific materials.

This may help my Project mates and me in our future careers in things like making decisions about what to study and even proposing new questions and problems to investigate. I have learnt plenty of techniques like how to prepare samples before the analysis of ion concentration.

If someone asked me if I would like to participate in this project again, I would say yes without hesitation.

*Este proyecto nos ha enseñado a trabajar como un científico así como a pensar al igual que uno, organizarme como tal y a trabajar en un laboratorio con muchos materiales científicos.*

*Esta participación seguro que nos ayudará a todos en un futuro en cosas como qué queremos estudiar o incluso en plantearnos nuevos problemas y preguntas que desarrollar sobre este proyecto. He aprendido muchas técnicas como cómo preparar una muestra antes del análisis de la concentración de iones.*

*Si alguien me preguntase si volvería a participar en este proyecto, afirmarí sin pensármelo dos veces*

**Ángela Maldonado Ortega**, CDP Sagrado Corazón de Jesús, Granada.

I have found the project PIIISA very positive because it has helped me to understand the research world and it has also given me theoretical and practical knowledge, which is the result of daily teamwork with the help of professional researchers.

I think that this experience will help me in the future, so thank you for the trust you have placed in me by allowing me to participate in this research project.

*El proyecto PIIISA me ha parecido muy positivo porque me ha ayudado a conocer el mundo de la investigación además de aportarme conocimientos no sólo a nivel teórico, sino sobre todo*

*práctico, ya que el resultado obtenido es fruto del trabajo en equipo realizado día a día gracias a la ayuda de nuestros investigadores.*

*Pienso que esta experiencia me va ayudar mucho en el futuro, por lo que agradezco la confianza que han puesto en mí para hacerme partícipe de este proyecto de investigación.*

**Patricia Vílchez Fernández**, IES Montes Orientales, Iznalloz, Granada

In this project, we have had the opportunity to experiment for ourselves how scientific work is carried out and how to be a real researcher. During these months, with the help of professionals, we have learnt how to carry out an investigation.

We have had, for the first time, the chance to work in a laboratory, using the same resources and methods that researchers use normally. We also learnt to be patient and reliable to go on with the investigation successfully. This project has introduced us to the world of research, and enables us to clarify our future career expectations.

We now know how important research is for our lives and all the things it can give us to improve them.

*Con este proyecto hemos tenido la oportunidad de experimentar por nosotros mismos como es el trabajo científico y ser como verdaderos investigadores. Durante estos meses, con la ayuda de profesionales, hemos aprendido cómo llevar a cabo una investigación.*

*Por primera vez hemos podido trabajar en un laboratorio, usando los mismos recursos y métodos que los investigadores utilizan normalmente. También aprendimos a ser pacientes y responsables para llevar a cabo una investigación con éxito. Este proyecto nos ha introducido en el mundo de la investigación, por lo que podemos ver más claramente nuestras expectativas para el futuro.*

*Ahora sabemos lo importante que es la investigación para nuestras vidas y todas las mejoras que nos aporta.*

**Encarnación Pérez Santiago**, CDP Virgen de Gracia, Granada.

In this project, we have seen how researchers work in their laboratories and we have also had the opportunity to work with them. We have worked with some techniques we did not know such as growing plants in different mediums (pots, hydroponics and Petri dishes) as well as crushing plants in order to weigh them.

We also have learnt the importance of being patient and careful in a project and of not giving up if something goes wrong. And we have participated in writing the proceedings and in all the work involved.

So now, we have more information to think about our future careers.

*En este proyecto hemos visto cómo trabajan los investigadores en sus laboratorios y también, hemos tenido la oportunidad de trabajar con ellos. Hemos trabajado con algunas técnicas que no conocíamos, moler las plantas para pesarlas y plantarlas en diferentes medios, como macetas o placa Petri.*

*También hemos aprendido la importancia de ser pacientes y cuidadosos en un proyecto y no rendirse si algo sale mal. Hemos llevado a cabo el proceeding y las tareas.*

*Así que ahora, tenemos más información para pensar en nuestro futuro.*

**Ana López López**, IES ACCI, Guadix Granada.

In this project, we have seen how salt affects two types of tomatoes, with two different genes transporting sodium and we have cultivated this tomato plants in three different types of cultivation, for example, in pots, hydroponic systems and Petri plates. In my opinion, this project is a good idea because, apart from learning new concepts, it teaches us how to work in

laboratories and how research is carried out by experts in this field. It was also interesting because I had never participated in any activity like this before and I have learned to use scientific tools.

I think that what I liked most was being able to participate in this project which I would not have had the opportunity to experience at my age anywhere else.

*En este proyecto hemos podido ver como la sal afecta a las plantas, en este caso en dos tipos de tomate para dos genes que se encargan de transportar el sodio en las plantas y nosotros hemos cultivado estos tomates en diferentes tipos de cultivo, como por ejemplo, placas petri, macetas y en sistema hidropónico. En mi opinión, este proyecto es una buena idea porque aparte de enseñarnos nuevos conceptos, nos enseña a trabajar en laboratorio y a saber cómo se realizan los proyectos de investigación. Este proyecto me ha parecido también interesante porque yo nunca había participado en alguna actividad como ésta y me ha proporcionado la oportunidad de trabajar con nuevas herramientas.*

*Lo que más me ha impresionado es poder realizar esta experiencia a mi edad ya que en ningún otro sitio cercano podre realizarla.*

Thanks

We would like to thank all the research team made up of Noelia Jaime, Raquel Olías and Andrés Berver for all the help, time and support they have given us in order to make this project possible.